# AN *IN SILICO* EXPLORATION OF NOVEL ANTI-FIBROTIC AGENTS FOR WOUND HEALING

*A THESIS SUBMITTED FOR PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF*

**BACHELOR OF TECHNOLOGY**

**IN**

**BIOTECHNOLOGY**

**By**

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# CERTIFICATE

This is to certify that the project entitled, "**An *In Silico* Exploration of Novel Anti-Fibrotic Agents for Wound Healing**" submitted by **Soumya Ranjan Moharana** is an authentic work carried out by him under my supervision and guidance for the partial fulfillment of the requirements for the award of **Bachelor of Technology (B. Tech) Degree in Biomedical Engineering** at **National Institute of Technology, Rourkela.**

To the best of my knowledge, the matter embodied in the thesis has not been submitted to any other University/ Institute for the award of any Degree or Diploma.

**Date:**

**Place: Rourkela**

**Dr. Bibhukalyan Prasad Nayak**
**Dept. of Biotechnology & Medical Engineering**
**NIT Rourkela**

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Thank you.

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# ABSTRACT

A breach in epithelial lining from trauma or disease leads to wound formation. Wounds can vary in depth and can be incised, chopped, lacerated or a complex type depending upon the inducing agent and the direction of force applied. An untreated wound follows the natural course of healing that comprises of four phases. Prior to remodeling, the final phase in wound healing, proliferation phase initiates when the fibroblasts start depositing new extracellular matrix. Sometimes, the remodeling is compromised and there is excessive matrix deposition. This phenomenon is termed fibrosis. A number of signal transduction pathways orchestrate the wound healing and a disparity in the interaction leads to fibrosis. Transforming Growth Factor Beta (TGF-β) pathway acts as a hub in the whole interaction of wound healing and an abnormal response in its receptor has been confirmed to be the root cause of fibrosis. In the current project, TGF-β receptor was investigated as the target protein to alleviate fibrosis using bioinformatics tool. Briefly, the structures of TGF-β receptors were retrieved from PDB. A series of natural and synthetic drugs were searched and the corresponding structures were retrieved from PUBCHEM database. Ligand-receptor docking was done using Swiss-dock software. The structure of best docked ligand i.e. α-tocopherol was modified in ChemSketch followed by docking. Both virgin and the modified α-tocopherol can act as novel drug against excessive fibrosis; however it needs final validation *in vitro* and *in vivo*.



***Key Words –*** Wound; Fibrosis; TGF-β; Bioinformatics tools; Docking

# INTRODUCTION

# 1. INTRODUCTION

A wound occurs when there is injury to a tissue (e.g. skin break, muscle tear/burn or a bone fractures). Wounds are the outcome of external physical trauma. The most common causes of wounds are vehicle accidents, falls and the misuse of sharp objects, tools, machinery, weapons, a surgical procedure, an infectious disease or an underlying pathological condition. [1]

### 1.1. Types of Wound

Wounds are classified as open or closed. An open wound is a break in the skin or in a mucous membrane. A closed wound involves underlying tissues without a break in the skin or a mucous membrane. Wounds are of various types and have different causes, based on which there are several ways of classifying them. [2]

Wounds are of the following types: -

- Abrasions (scrapes) – skin rubbed away due to frictional force. [1]
- Avulsions – forced removal of tissue from body. [1]
- Lacerations – blunt and irregular breaks due to use of high force. [1]
- Punctures – narrow and deep wound due to penetration of sharp objects like a pin. [1]
- Incisions – sharp cut due to sharp instrument. [1]
- Contusions – internal injury due to forced trauma. [1]

### 1.2. Mechanism of Wound Healing

Wound healing is a continuously changing, difficult to understand phenomenon in which the wounded area changes with respect to the health of the individual. The idea about normal wound healing process through the phases of hemostasis, inflammation, granulation and maturation provides a framework for an understanding of the basic principles of wound healing. Through this understanding the health care professional can develop the skills required to care for a wound and the body can be assisted in the complex task of tissue repair. [2]

A chronic wound should prompt the health care professional to begin a search for unresolved underlying causes. Healing a chronic wound requires care that is patient centered, holistic, interdisciplinary and cost-effective and evidence based. [2]

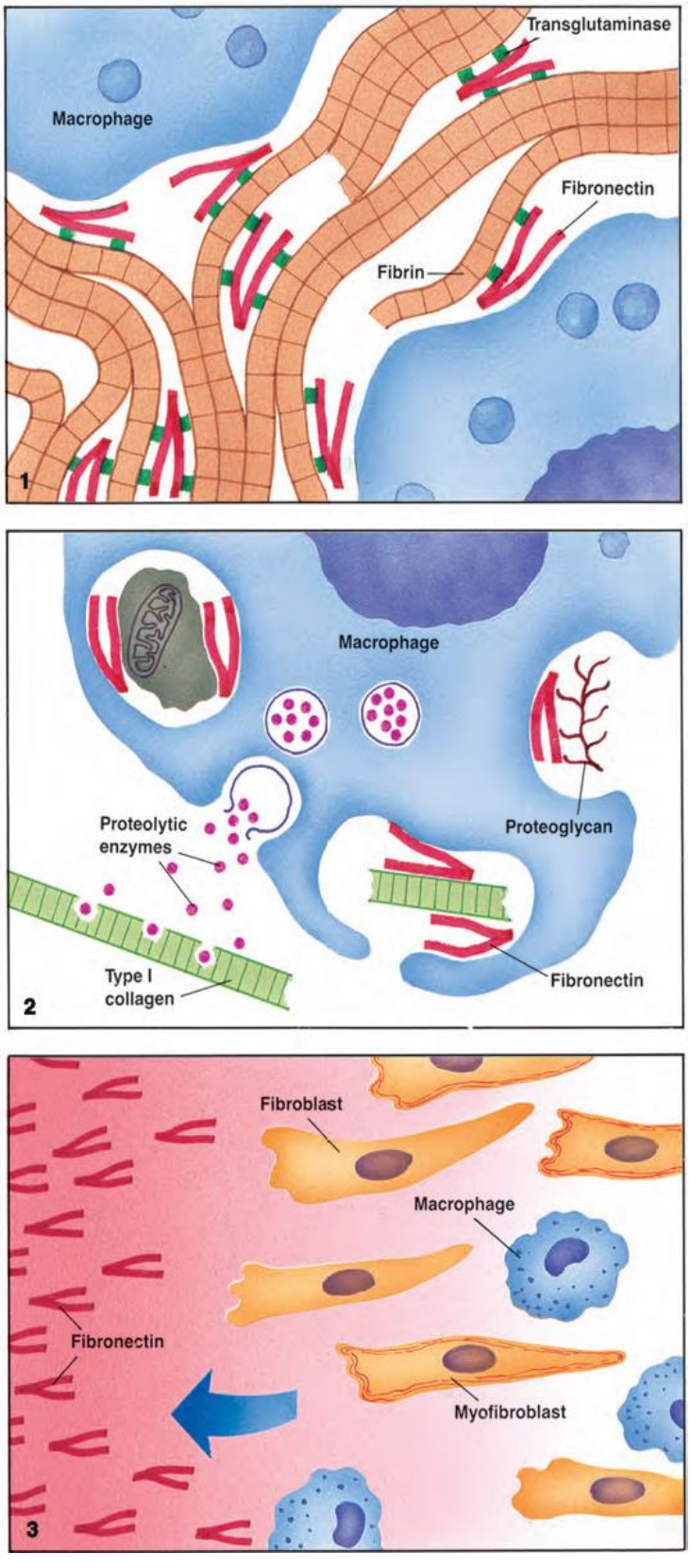
### 1.2.1. *Phases of Wound Healing*

Based on research, both acute wounds and heals chronic wounds follow four basic phases [2] as described below: -

- **Hemostasis –** During an injury, healing process starts as blood platelets at the site of injury comes in contact with extracellular matrix which leads to the release of growth factors and cytokines such as platelet-derived growth factor (PDGF) and transforming growth factor beta (TGF-ß). [3]
- **Inflammation –** The neutrophils as well as the macrophages come to the site and remove the foreign materials, microbes and damaged tissues by phagocytosis releasing more PDGF and TGF-ß, paving the way for proliferation phase to start. [3]
- **Proliferation or Granulation –** After cleaning of the wound site fibroblasts reach the wound site and deposit new extracellular matrix. [3]
- **Remodeling or Maturation –** The new collagen matrix becomes cross-linked and organized during this stage. [3]

### 1.2.2. *Signal Transduction Pathways in Wound Healing*

There are numerous signaling pathways involved in the complicated and dynamic, repairing process of wound healing. During wound healing, by the time Proliferation stage starts, fibroblasts have started depositing extracellular matrix. *Fibrosis* is the result of the surplus expression of extracellular matrix by fibroblast during that stage. The extracellular matrix deposited is mostly composed of collagen expressed by the reaction of different growth factors - TGF-β, PDGFs, IGF, CTGF and TNF's. But the Inhibition of collagen secretion is dependent on TGF-β. In this study the main receptors of TGF-β pathway were studied and various natural and currently used synthetic drugs were used to determine their binding with the receptors, which could lead to the finding of a new drug for the treatment of fibrosis.

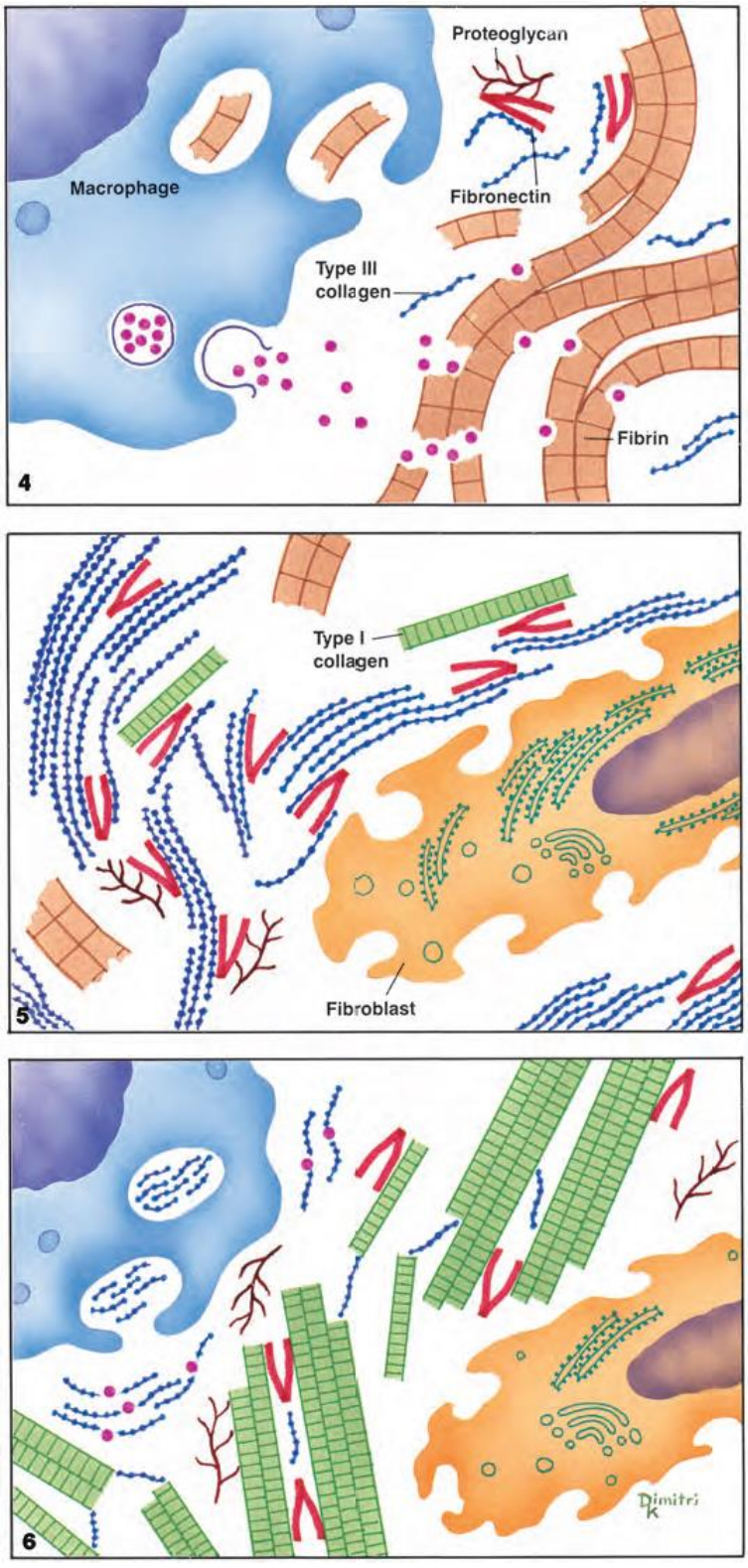



The initial phase of the repair reaction typically begins with hemorrhage into the tissues.

**(1)** A fibrin clot forms and fills the gap created by the wound. Fibronectin in the extravasated plasma is cross-linked to fibrin, collagen, and other extracellular matrix components by the action of transglutaminases. This cross-linking provides a provisional mechanical stabilization of the wound (0–4 hours).

**(2)** Macrophages recruited to the wound aea process cell remnants and damaged extracellular matrix. The binding of fibronectin to cell membranes, collagens, proteoglycans, DNA and bacteria (opsonization) facilitates phagocytosis by these macrophages and contribute to the removal of debries.

**(3)** Fibronectin, cell debris, and bacterial products are chemo-attractants for a variety of cells that are recruited to the wound site (2–4 days).




The intermediate phase of the repair reaction;

**(4)** As a new extracellular matrix is deposited at the wound site, the initial fibrin clot is lysed by a combination of extracellular proteolytic enzymes and phagocytosis (2–4 days).

**(5)** Concurrent with fibrin removal, there is deposition of a temporary matrix formed by proteoglycans, glycoproteins, and type III collagen (2–5 days).

**(6)** Final phase of the repair reaction. Eventually the temporary matrix is removed by a combination of extracellular and intracellular digestion, and the definitive matrix, rich in type I collagen, is deposited (5 days–weeks).

**Figure -1: -Summary of the healing process** [5]

# 2. LITERETURE REVIEW

## 2.1. Transforming Growth Factor β (TGF-β) and Fibrosis:

Transforming growth factor beta (TGF-β) is a cytokine that mediates cellular differentiation by controlling signal transduction pathways. In addition it has an wide range of functions that include immune function, oncogenesis and the pathophysiology of heart disease, diabetes, Marfan syndrome.

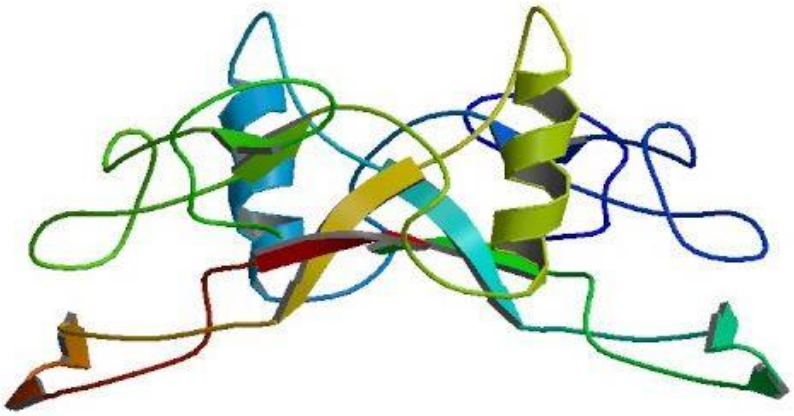


**Figure 2 - Solution structure of TGF-β1**

TGF-β has three isoforms called TGF-β1, TGF-β2 and TGF-β3. TGF-β acts as tumor suppressive factor at early stages of oncogenesis. Some cells secrete TGF-β secreted by some cells can act on the same cell through the expression of its own receptor. Such autocrine signaling increases the production of TGF-β, which also acts on the surrounding cells.

## 2.2. TGF-β ACTIVATION

TGF-β ligand upon activation will initiate the TGF-β signaling cascade depending upon the availability of TGF-β receptors I and II due to high affinity between TGF-β and its receptors. Thus, TGF-β signaling recruits a latency system for cell signaling. [4] Such activation of TGF-β is found to be dependent on reactive oxygen species, pH and proteases.

## TGF-β Pathway

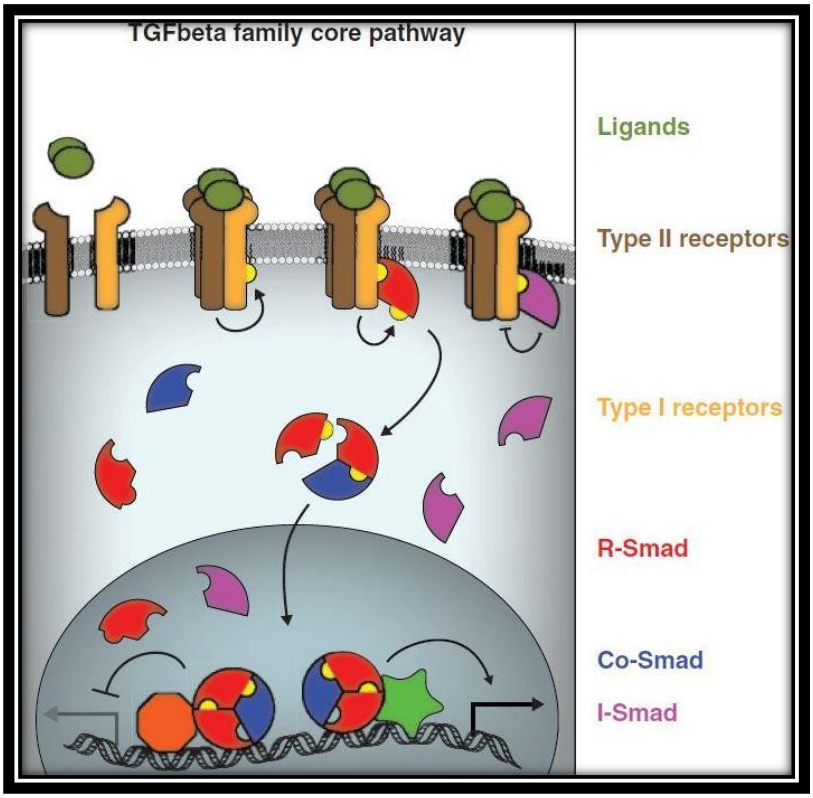



**Figure 3 – TGF-β Family Core Pathway** [6]

Transforming growth factor (TGF) beta superfamily comprises of over 30 members including Activins, Nodals, Bone Morphogenetic Proteins (BMPs), and Growth and Differentiation Factors (GDFs). The signaling of the TGF–β superfamily is through ligand binding to the serine/threonine kinase receptors which gives signal to Smad protein, and the gene expression in the nucleus starts. [6]

Signals for TGF-beta superfamily members are transmitted through heteromeric complexes comprised of type I and II trans-membrane serine/threonine kinase receptors. [6]

## 2.3. TGF-B receptors

- ***TGF-β Receptor, Type I***

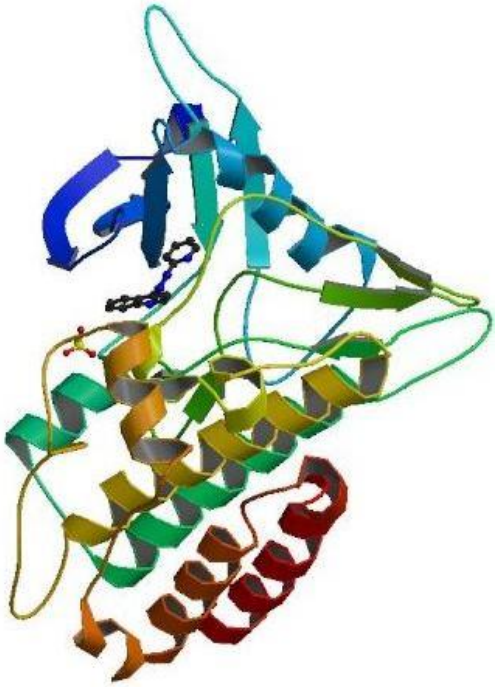


**Figure-4: - Crystal Structure of TGF-beta receptor I kinase with inhibitor (1PY5)**

- ***TGF-β Receptor, Type II***

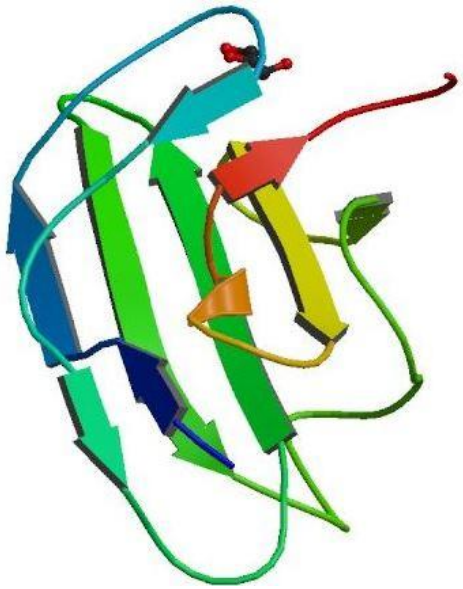


**Figure-5: - Crystal Structure of Human TGF-Beta type II receptor ligand binding domain (1m9z)**

# TOOLS AND METHODOLOGY

# 3.1. Tools used

**Swiss PDB Viewer – "**http://www.expasy.org/spdbv"

To view the PDB structures of the compounds and ligands and for energy minimization. For energy minimization the following steps were followed –

- In menu bar – Select > all
- Tools > energy minimization
- Ctrl + S > save as "own required file name"

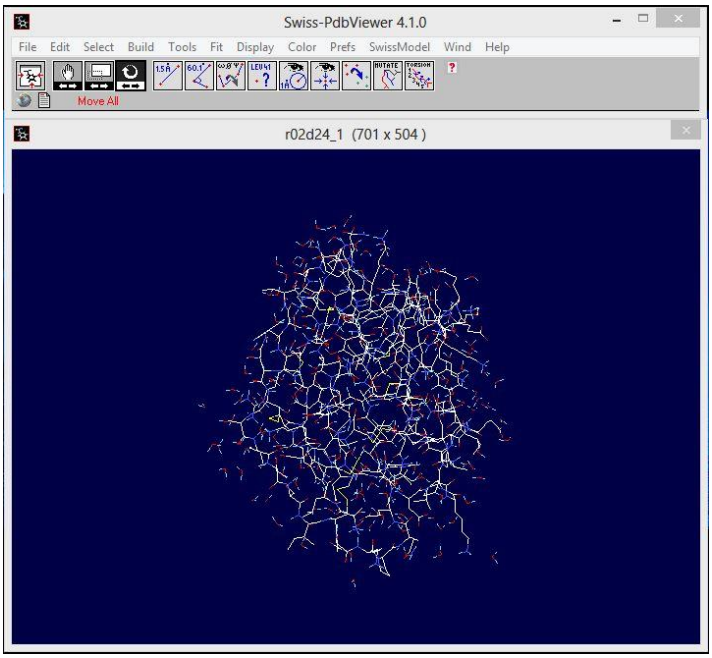



**Figure 6 – SWISS PDB VIEWER**

**PUBCHEM – "**http://pubchem.ncbi.nlm.nih.gov/"

This database is a collection of cross-checked molecule structures which was used to retrieve all the ligands used in this study.


**Figure 7 - PUBCHEM**

**Protein Data Bank** – "http://www.rcsb.org/pdb/home/home.do"

Online database of biological macromolecules found worldwide which was used to retrieve the structure of the receptor molecules.


**Figure 8 – Protein Data Bank**

**ARGUS LAB**

Molecular viewer software used to view edit and dock ligands to the receptors. It was used to optimize the geometry of modified molecule.

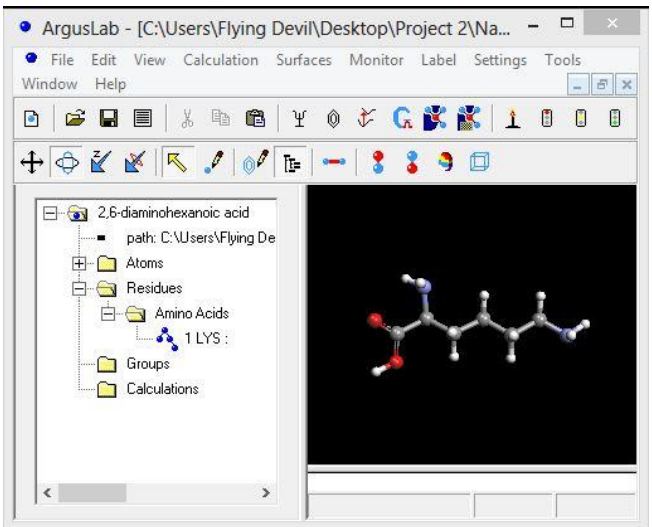



**Figure 9 – Argus lab**

**UCSF CHIMERA**

Powerful molecular viewer software providing data on docked structure by use of VIEWDOCK extension. This software was used to view the *.chimerax*_extension file provided by the SWISSDOCK (*on-line docking server)* which provided the Binding energy results.

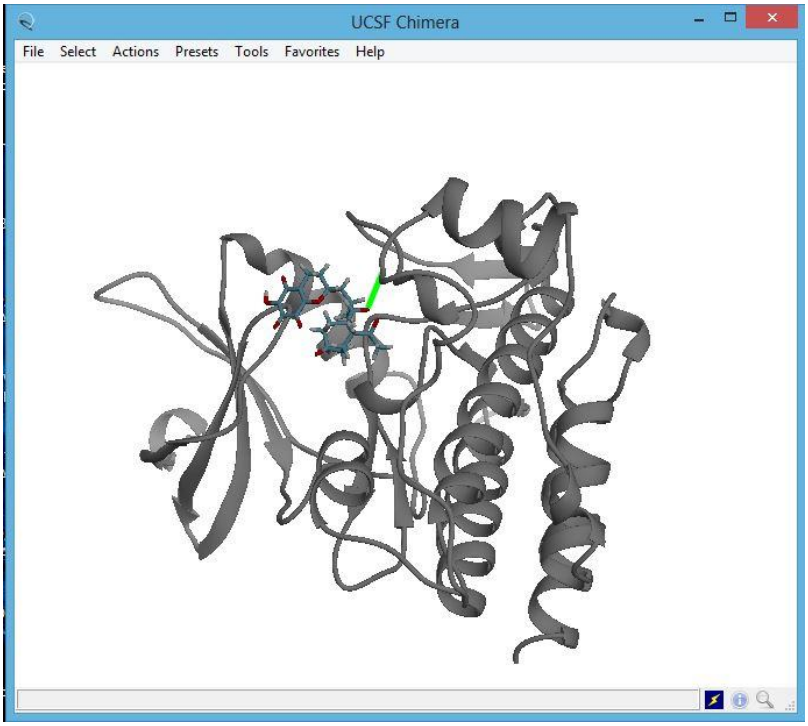



**Figure 10 - UCSF CHIMERA**

**CHEMSKETCH**

Software used to draw chemical structure easily and calculate their properties. This software was used to remove the $CH_3$ groups and insert OH groups instead to create synthetic drugs for testing.

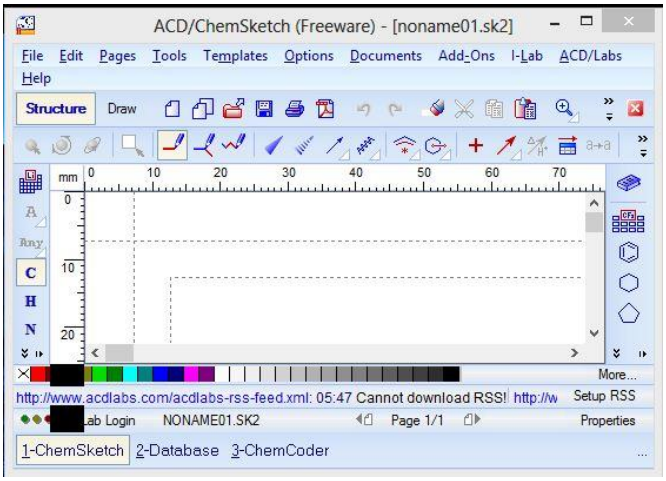



**Figure 11- CHEMSKETCH**

**SWISSDOCK ONLINE DOCKING – "**http://swissdock.vital-it.ch/docking"

This online docking server was used to dock the selected ligands with the receptors and provided Binding energy results.

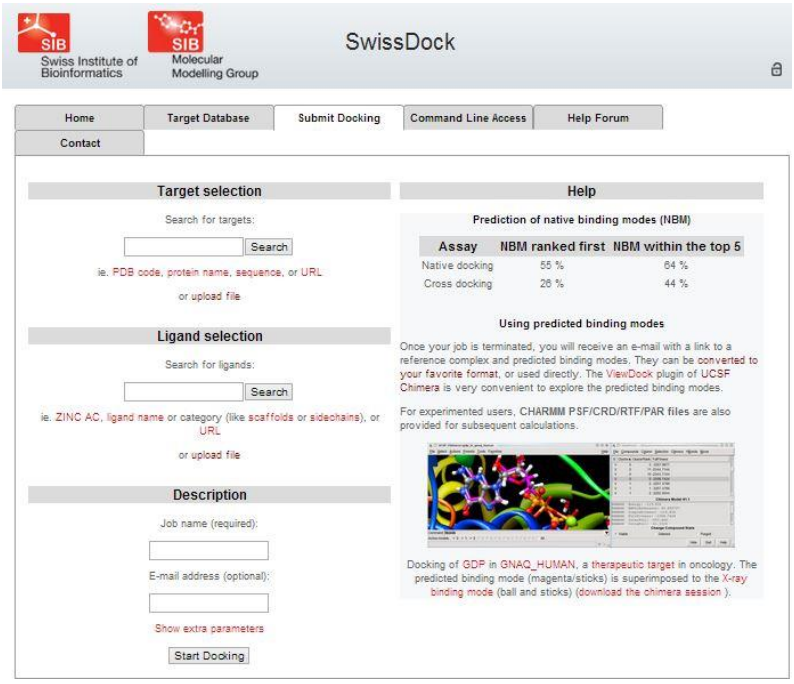



**Figure 12- SWISSDOCK online docking**

**OPEN BABEL**

Software used for converting various molecular file formats, example – converting *.mol* file to *.pdb* file.

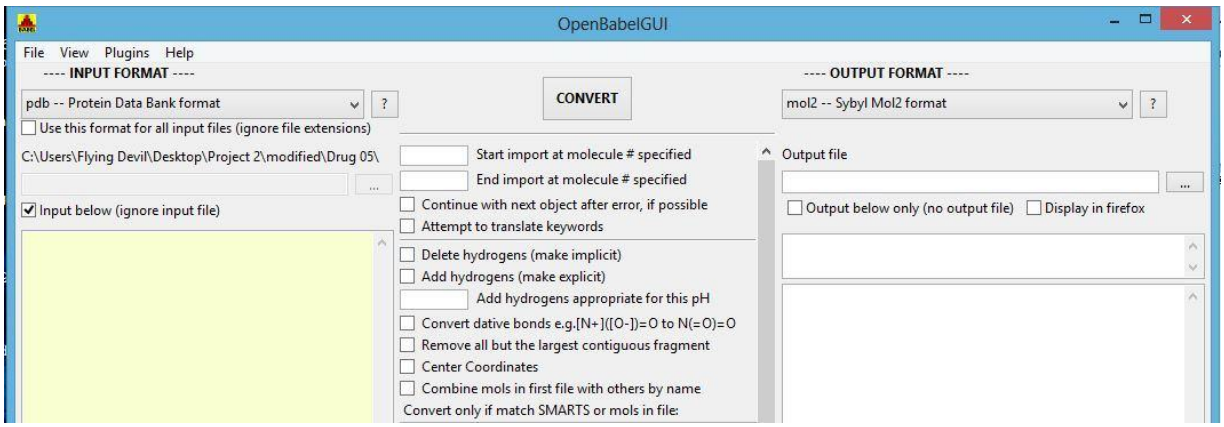
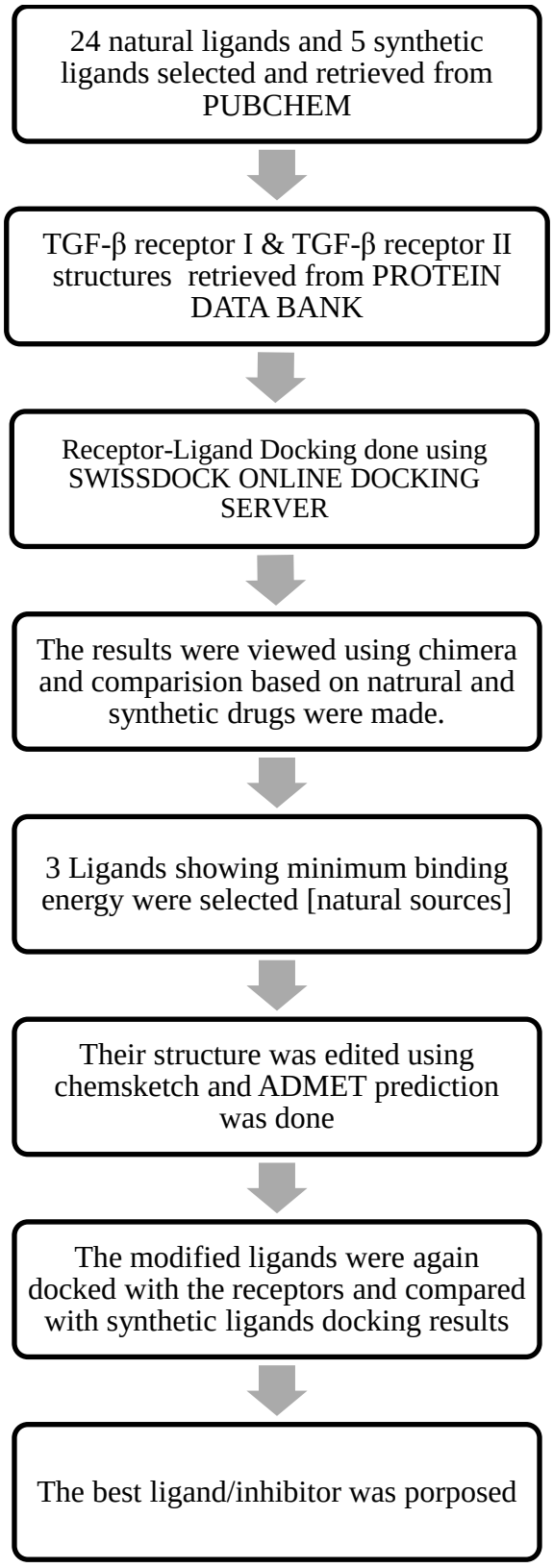



**Figure 13 - BABEL**

# 3.2. Plan of Work



## 3.3. Procedure

**1. Structure Retrieval –**

All the structures of drugs/ligands were retrieved from PUBCHEM and conversion to required file format was done using BABEL.The structures of the receptors were retrieved from PROTEIN DATA BANK in the form of *.pdb* file format.

## 2. Docking –

The downloaded ligand files were docked with the receptor to find the best ligand with most stability, i.e. minimum binding energy. The docking was done using SWISS DOCK ONLINE SERVER.

**3. Selection of Ligands –**

From the binding energies obtained, three molecules with the lowest binding energy were selected (from the 24 natural drugs) as natural treatment for fibrosis.

**4. Ligand Modification and ADMET prediction–**

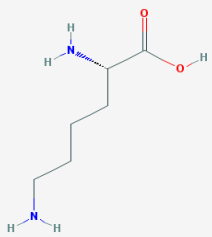
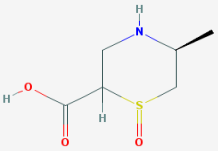
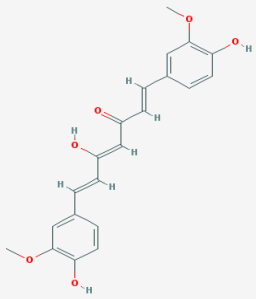
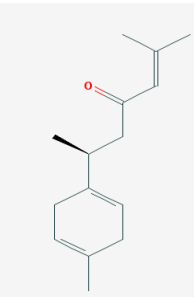
The three molecules were then modified using CHEMSKETCH by removing their methyl groups and adding oh group at that place. These modified drugs were then placed under ADMET prediction for *Aerobic Biodegradability*, *Ames Mutagenicity*, *Developmental Toxicity Potential* and *Ocular Irritancy Mild vs. Moderate Severe.*

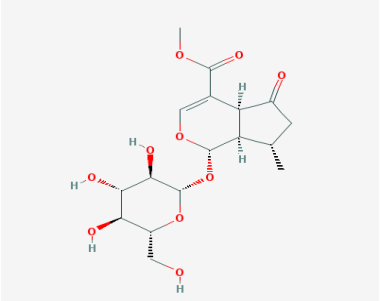
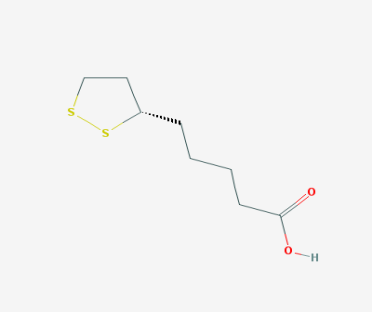
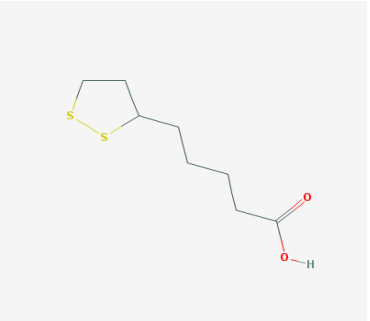
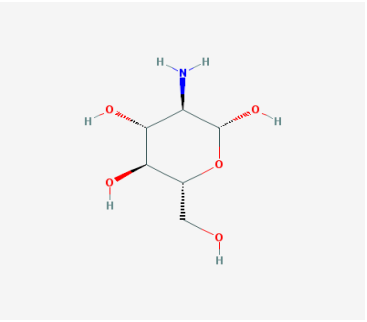
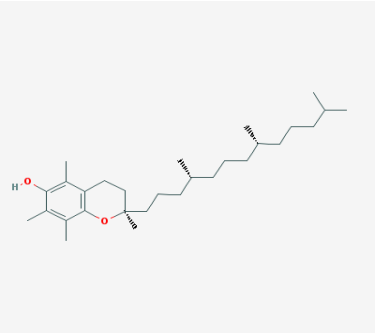
**5. Docking with Modified Ligand –**

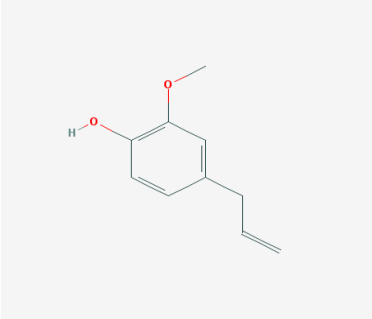
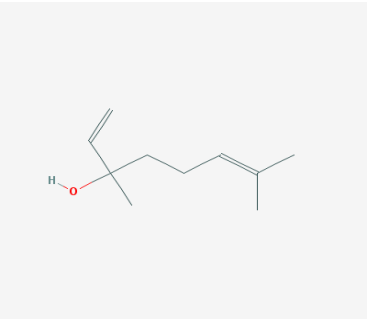
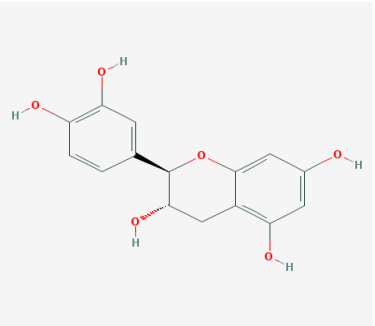
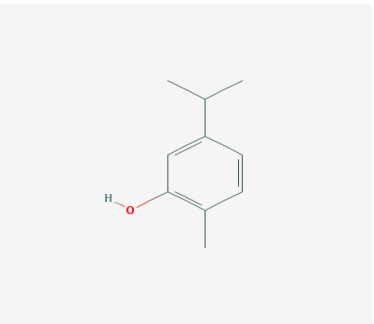
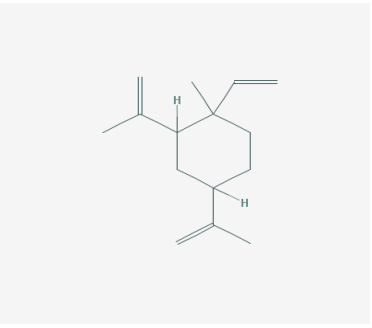
From the modified drugs the drug with the lowest toxicity was selected and docked with the receptors to find its binding energy with the receptors.

## 3.4. List of Drugs Used

### Table 1: List of Natural Ligands of TGF-β receptors

| Name of Drug (Natural Ligand) | Pubchem (i.d.) | Molecular Weight | Molecular Formula | Structure |
|---|---|---|---|---|
| **Lysine** | CID 5692 | 146.18756 | $C_6H_{14}N_2O_2$ |  |
| **Cycloallin** | CID 193294 | 177.22144 | $C_6H_{11}NO_3S$ |  |
| **Curcumin** | CID 5281767 | 368.3799 | $C_{21}H_{20}O_6$ |  |
| **Turmerone** | CID 14367555 | 218.33458 | $C_{15}H_{22}O$ |  |

| | | | | |
|---|---|---|---|---|
| **Verbenalin** | CID 73467 | 388.36646 | $C_{17}H_{24}O_{10}$ |  |
| **Alphalipoic acid** | CID 445125 | 206.32556 | $C_{15}H_{22}O$ |  |
| **1,2 – Dithiolane -3- Valric** | CID 864 | 206.32556 | $C_8H_{14}O_2S_2$ |  |
| **Glucosamine** | CID 441477 | 179.17112 | $C_6H_{13}NO_5$ |  |
| **Alpha tocopherol (Vitamin E)** | CID 14985 | 430.7061 | $C_{29}H_{50}O_2$ |  |

| | | | | |
|---|---|---|---|---|
| **Eugenol** | CID 3314 | 164.20108 | $C_{10}H_{12}O_2$ |  |
| **Linalool** | CID 6549 | 154.24932 | $C_{10}H_{18}O$ |  |
| **Catechin** | CID 9064 | 290.26806 | $C_{15}H_{14}O_6$ |  |
| **Carracrol** | CID 10364 | 150.21756 | $C_{10}H_{14}O$ |  |
| **Elemene** | CID 10583 | 204.35106 | $C_{15}H_{24}$ |  |

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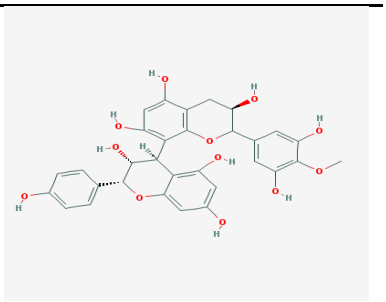
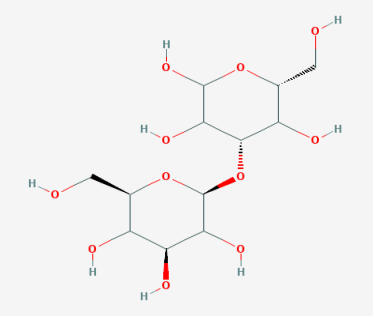
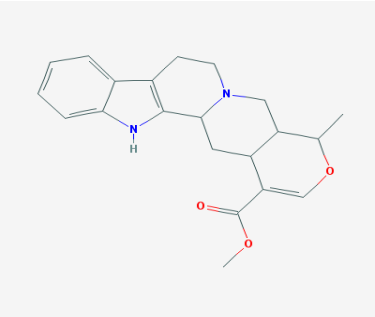
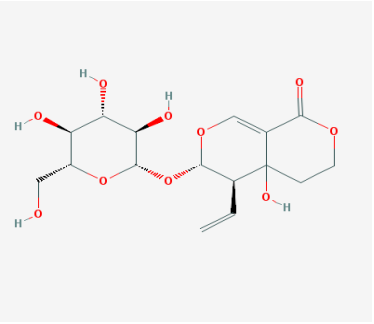
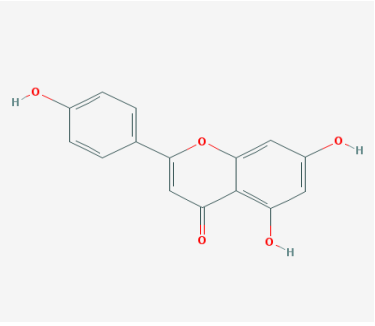
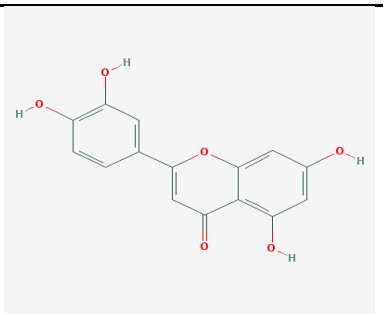
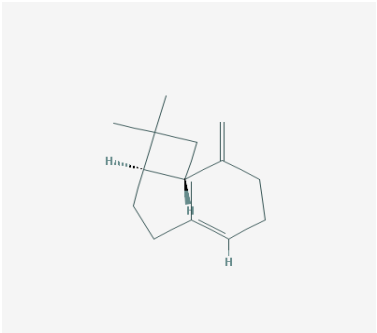
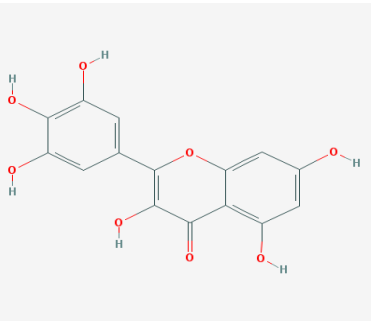
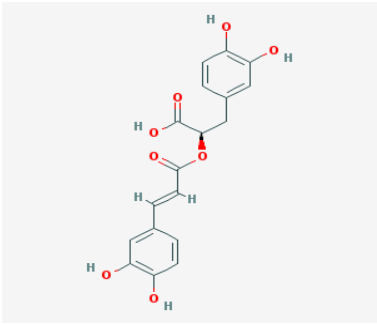
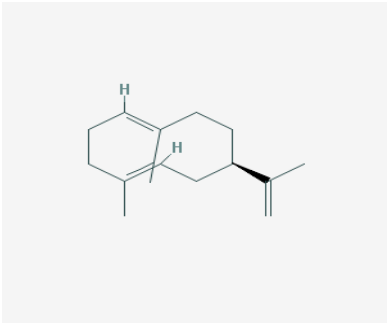
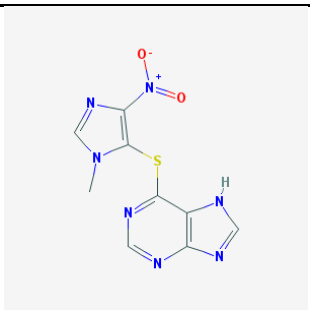
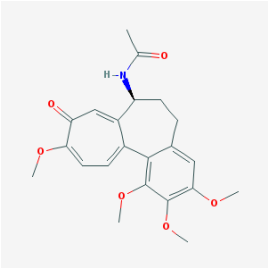
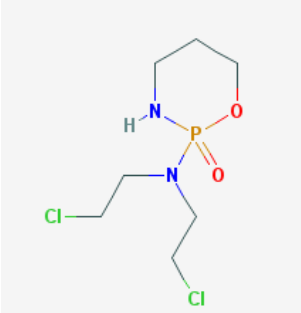
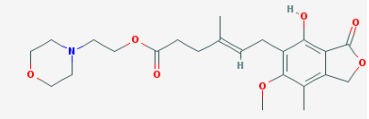
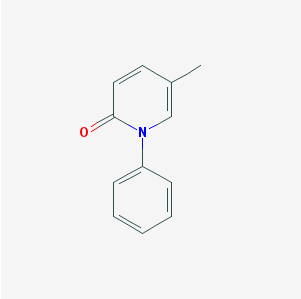
|---|---|---|---|---|
| **Proanthocyanidin A** | CID 108065 | 592.54682 | $C_{31}H_{28}O_{12}$ |  |
| **Laminaribose** | CID 25245814 | 342.29648 | $C_{12}H_{22}O_{11}$ |  |
| **Ajmalicine** | CID 251561 | 352.42686 | $C_{21}H_{24}N_2O_3$ |  |
| **Swertiamarin** | CID 114700 | 374.33988 | $C_{16}H_{22}O_{10}$ |  |
| **Apigenin** | CID 5280443 | 270.2369 | $C_{15}H_{10}O_5$ |  |

| | | | | |
|---|---|---|---|---|
| **Luteolin** | CID 5280445 | 286.2363 | $C_{15}H_{10}O_6$ |  |
| **Caryophyllene** | CID 5281515 | 204.35106 | $C_{15}H_{24}$ |  |
| **Myricetin** | CID 5281672 | 318.2351 | $C_{15}H_{10}O_8$ |  |
| **Rosmarinic Acid** | CID 5281792 | 360.31484 | $C_{18}H_{16}O_8$ |  |
| **Germacrene A** | CID 9548705 | 204.35106 | $C_{15}H_{24}$ |  |

## Table 2: List of Synthetic Ligands of TGF β receptors

| NAME OF DRUG(LIGAND) [NATURAL] | PUBCHEM ID | MOLECULAR WEIGHT | MOLECULAR FORMULA | STRUCTURE |
|---|---|---|---|---|
| **Azothioprine** | CID 2265 | 277.26258 | $C_9H_7N_7O_2S$ |  |
| **Colchicine** | CID 6167 | 399.437 | $C_{22}H_{25}NO_6$ |  |
| **Cyclophosphamide** | CID 2907 | 261.085962 | $C_7H_{15}Cl_2N_2O_2P$ |  |
| **Mycophenolate Mofetil** | CID 5281078 | 433.49474 | $C_{23}H_{31}NO_7$ |  |
| **Pirfenidone** | CID 40632 | 185.22184 | $C_{12}H_{11}NO$ |  |

# RESULTS AND DISCUSSIONS

## 4. Results and Discussions

### 4.1. Ligand (Unmodified)-Protein Docking Result

Receptor and ligand were docked using SWISSDOCK online server. The results obtained are: -

**Table 3: Binding energy of Natural Ligands Following Docking**

| Name of Drug (Natural Ligand) | ΔG (Kcal/Mol) Receptor 1 | ΔG (Kcal/Mol) Receptor 2 |
|---|---|---|
| **Lysine** | -7.0551524 | -5.9956436 |
| **Cycloallin** | -6.8762417 | -6.046295 |
| **Curcumin** | -9.070615 | -7.160752 |
| **Turmerone** | -6.8523955 | -6.253523 |
| **Verbenalin** | -8.317615 | -6.9071236 |
| **Alpha lipoic acid** | -7.7582974 | -6.269606 |
| **1,2 – Dithiolane -3- Valric** | -7.578595 | -6.4135394 |
| **Glucosamine** | -6.9926643 | -6.1596193 |
| **Alpha tocopherol (Vitamin E)** | -8.713398 | -6.3799357 |
| **Eugenol** | -7.01427 | -5.9118385 |
| **Linalool** | -6.5363007 | -5.932526 |
| **Catechin** | -8.092369 | -6.487141 |
| **Carracrol** | -6.768245 | -5.8302073 |
| **Elemene** | -6.8595595 | -6.1184897 |
| **Proanthocyanidin A** | -7.698849 | -6.7706394 |
| **Laminaribose** | -8.582201 | -6.893973 |
| **Ajmalicine** | -7.6328998 | -6.258801 |
| **Swertiamarin** | -8.659184 | -6.737429 |
| **Apigenin** | -7.9015365 | -6.3797035 |
| **Luteolin** | -8.022708 | -6.398905 |
| **Caryophyllene** | -7.0452323 | -5.9958153 |
| **Myricetin** | -7.99371 | -6.437615 |
| **Rosmarinic Acid** | -9.114415 | -7.218021 |
| **Germacrene A** | -6.4372272 | -5.961119 |

**Table 4: Binding energy of Synthetic Ligands Following Docking**

| Name of Drug (Synthetic Ligand) | ΔG (Kcal/Mol) Receptor 1 | ΔG (Kcal/Mol) Receptor 2 |
|---|---|---|
| **Azothioprine** | -7.630012 | -6.602702 |
| **Colchicine** | -6.9731503 | -6.1768827 |
| **Cyclophosphamide** | -7.2774844 | -6.303278 |
| **Mycophenolate Mofetil** | -9.018024 | -6.849458 |
| **Pirfenidone** | -6.868995 | -6.028638 |

The Three molecules with best binding energy are

**Table 5: Binding Energy Of Best Three Ligands**

| Name of Drug (Natural Ligand) | ΔG (Kcal/Mol) Receptor 1 | Name of Drug (Natural Ligand) | ΔG (Kcal/Mol) Receptor 2 |
|---|---|---|---|
| **Rosmarinic Acid** | -9.114415 | **Rosmarinic Acid** | -7.218021 |
| **Curcumin** | -9.070615 | **Curcumin** | -7.160752 |
| **Alpha tocopherol (Vitamin E)** | -8.713398 | **Verbenalin** | -6.9071236 |

On Comparing Binding energy values of the selected drugs with those of synthetic drugs it was found that the selected (best 3) natural drugs have much better binding energy than those of the synthetic drugs. These drugs can be selected as inhibitor for TGF-β pathway.

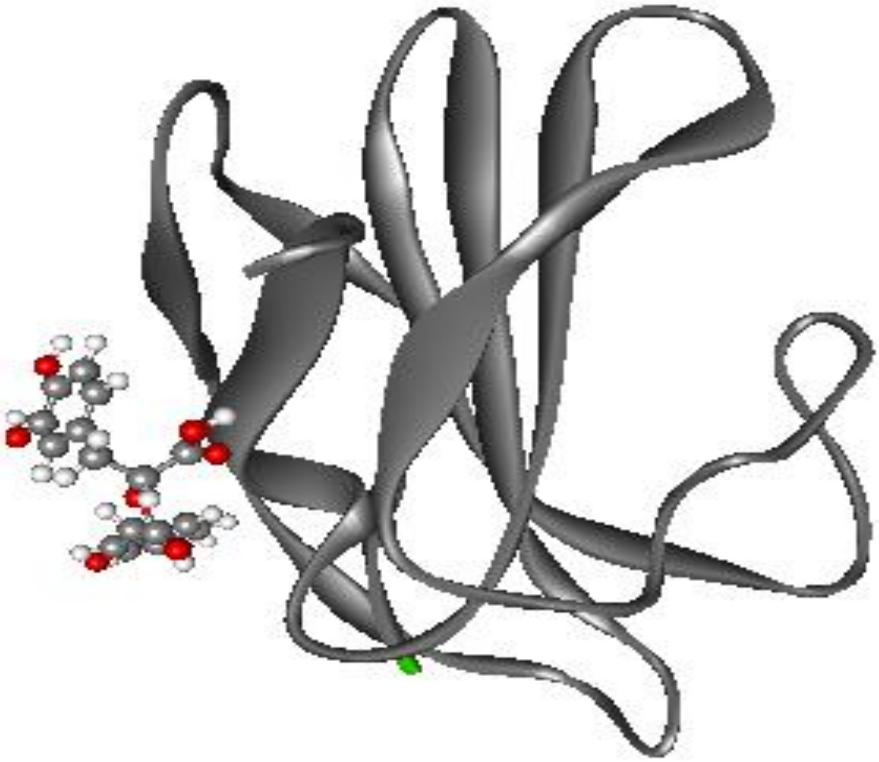


**FIGURE 14 – Rosmarinic Acid docked with Receptor II**

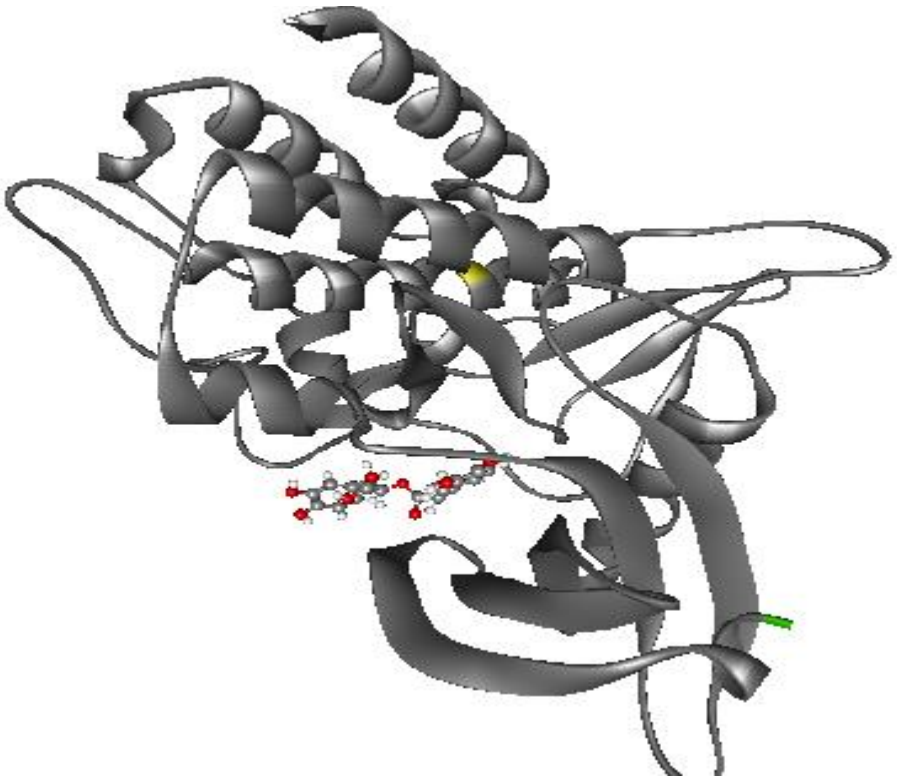


**FIGURE 15 - Rosmarinic Acid docked with Receptor 1**

### 4.2. Ligand Modification and Toxicity Testing

The drugs **Curcumin**, **Alpha Tocopherol** & **Verbenalin** were selected for modification. They were modified by removing Methyl group and adding OH group instead using chemsketch.

The toxicity of the modified molecules was tested using ADMET predictions.

**ADMET Prediction**

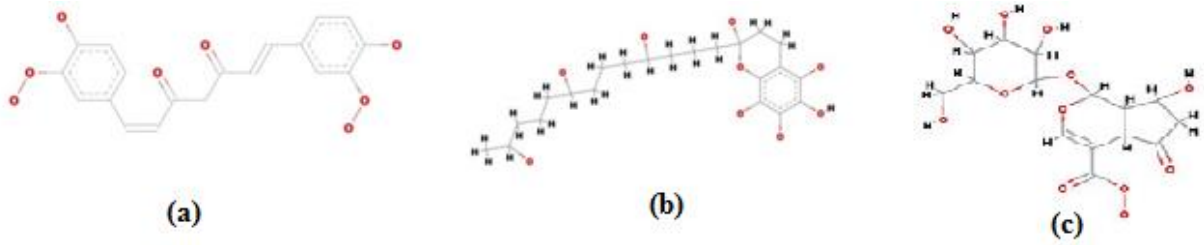



**Figure 16- Modified Ligands**

**Table 6: The Toxicity Report of Ligands from ADMET Server**

| Molecule | Modified Structure | Biodegradability | Toxicity | Mutagenicity | Ocular Irritancy |
|---|---|---|---|---|---|
| **Curcumin** | Fig.16(a) | Residues are degradable | Residues are highly toxic | Residues are non-mutagenic | Highly irritant |
| **α-tocopherol** | Fig.16(b) | Residues are degradable | The prostaglandin residue is non-toxic | Residues are non-mutagenic | Irritant **(cannot be used for eye scars.)** |
| **Verbenalin** | Fig.16(c) | Residues are degradable | All residues are toxic | One residue is non-mutagenic | Highly Irritant |

Since, **Alpha Tocopherol (**modified**)** was the most favorable one it was selected for docking with both the receptors.

### 4.3. Ligand (modified)-Protein Docking Result

After docking the results are as follows: -

With Receptor 1- ΔG = -15.824196 Kcal/Mol

With Receptor 2 - ΔG = -13.08529 Kcal/Mol

Thus, this drug is providing better binding energy results than the natural and synthetic drugs considered above. It can be used to treat fibrosis, but prior to that *in-vitro* testing should be done.

*Docked Structures [Ligand (modified)-Protein]*

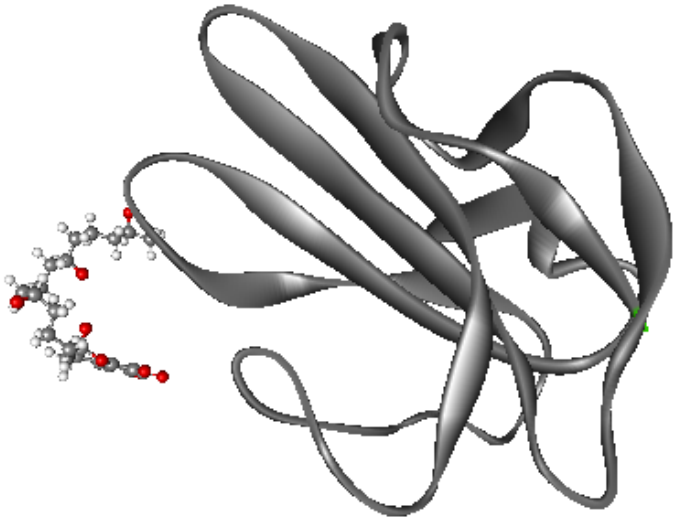


**Figure 17 –Vitamin E (modified) docked with Receptor II**

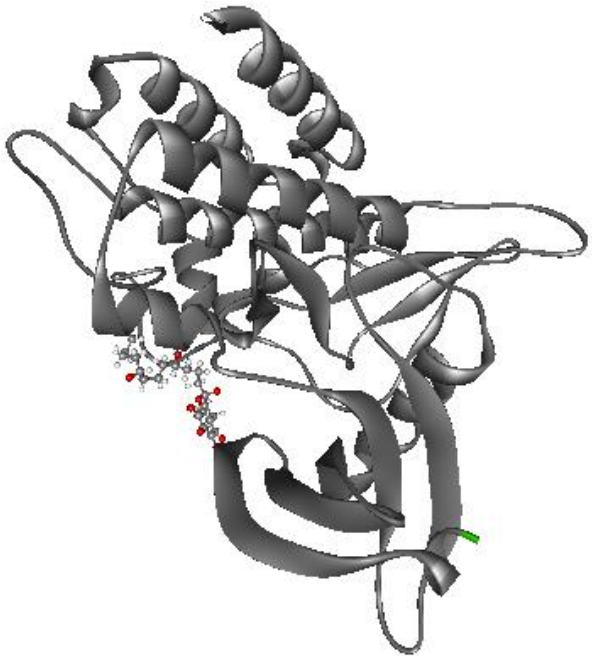


**Figure 18– Vitamin E (modified) docked with Receptor 1**

# CONCLUSION AND FUTURE WORK

## Conclusions

The natural drugs were tested under *in silico* condition and a drug with better binding result was shown as compared to the synthetic drugs. On the other hand, modifying it provided with a drug with better result. Hence, it can be said that *in silico* testing should be done for better results both capital and time are saved.

Base on the *in silico* experimentation the following drugs can be used as potential novel drugs for the prevention of fibrosis –

Natural - Rosmarinic Acid

ΔG (Kcal/Mol) Receptor 1= -9.114415 Kcal/Mol

ΔG (Kcal/Mol) Receptor 2= -7.218021 Kcal/mol

Synthetic - Mycophenolate Mofetil

ΔG (Kcal/Mol) Receptor 1= -9.018024 Kcal/mol

` ΔG (Kcal/Mol) Receptor 2= -9.018024 Kcal/mol

Modified - Alpha-tocopherol (modified)

ΔG (Kcal/Mol) Receptor 1= -15.824196 Kcal/Mol

ΔG (Kcal/Mol) Receptor 2= -13.08529 Kcal/Mol

However, before formulating an effective drug the above molecules need both *in vitro* and *in vivo* validation.

## Future Work

Future work could be the *in-vitro* preparation as well as testing of the drugs on fibrotic cells before making any conclusions about how good the drug is. This may include culturing of fibrotic cells and testing of the drugs on them.

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